

Mitochondrial Membrane Potential: A Novel Biomarker of Oxidative Environmental Stress

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Epidemiologic analyses, traditionally based on long-term cohort or case-control studies, provide retrospective causal associations between exposure to a particular environmental stressor and an exposure-related disease end point. Recent research initiatives have propelled a shift toward exploring molecular epidemiology and molecular biological markers (biomarkers) as a means of providing more immediate, quantitative risk assessment of potentially deleterious environmental exposures. We compared, in normal human monocytes isolated from the blood of healthy donors, variations in Hsp70 expression and mitochondrial membrane potential ($\Delta\psi_m$) in response to exposure to either tobacco smoke or γ -irradiation, two models for environmentally mediated oxidant exposure. On the basis of its mechanistic specificity for oxidants and little baseline variation in cells from distinct individuals, we propose that $\Delta\psi_m$ represents a selective *in vitro* and *in vivo* biomarker for oxidant exposure. $\Delta\psi_m$ may be used to gauge risks associated with oxidant-mediated air pollution and radiation. **Key words:** biomarker, environmental exposure, gamma-radiation, heat-shock proteins, Hsp70, mitochondrial membrane depolarization, oxidant, tobacco smoke. *Environ Health Perspect* 110:301–305 (2002). [Online 14 February 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p301-305vayssier-taussat/abstract.html>

Risk assessment is used to estimate the magnitude, likelihood, and uncertainty of environmentally induced toxic effects. Epidemiologic analysis, traditionally based on long-term cohort or case-control studies, provides retrospective causal associations between exposure to a particular environmental stressor and an exposure-related disease end point. Recent research initiatives have propelled a shift toward exploring molecular epidemiology and molecular biological markers (biomarkers) as a means of providing more immediate, quantitative risk assessment of potentially deleterious environmental exposures (1–4).

Three categories of biomarkers have been identified: biomarkers of effect, which are cellular responses that reflect sublethal exposure-related damage to a system; biomarkers of exposure, which are reversible upstream markers that respond before cellular damage occurs; and biomarkers of susceptibility, which refer to individual variations in the genes coding for stressor-induced cellular response (5). Proteins whose genes are influenced and induced by environmental stimuli or ecologic variations are called ecoproteins, in contrast to the constitutive, structural, “eco-free” proteins. Ecoproteins, which are generally highly inducible and conserved in nature, represent protective mechanisms against environmental stress and amplify the system’s ecophysiological adaptation to environmental conditions.

Among ecoproteins, stress proteins have been abundantly studied as a biomarker of effect for pollutants (6,7). Heat-shock proteins

(HSPs) represent the most abundant and widely studied group of stress proteins. HSPs are induced as an adaptive response on exposure to a variety of cellular injuries including oxidative damage. In particular, the cytosolic, inducible, 72 kDa HSP (Hsp70) is induced by oxidants both *in vitro* and *in vivo* (8–10), and its expression has been used as an indicative response to environmental stress and an interesting candidate as biomarkers of effect (11–14). Optimal biomarkers would be biomarkers of exposure, which could provide the earliest, most upstream warning signs of environmental stress exposure. Mitochondria are highly sensitive to oxidants and to toxic exposure-mediated cell death (15–17). We have previously shown that mitochondria were a target for reactive oxygen species-mediated effects of *in vitro* tobacco smoke (TS) exposure in human monocytes (18). We thus hypothesized that mitochondrial alterations, as determined by mitochondrial membrane potential ($\Delta\psi_m$), could represent a primary target for oxidant toxicity and could be used as a specific biomarker for oxidant-mediated exposure.

In this study, we first compared the variability of baseline $\Delta\psi_m$ to baseline Hsp70 expression in human monocytes isolated from the blood of healthy donors. We then analyzed the effects of two oxidant-mediated environmental stressors, TS and γ -radiation, on $\Delta\psi_m$. Our data suggest that the inherent variability of baseline levels of Hsp70 detected in human cells renders Hsp70 expression difficult to use as a biomarker for *in vivo* exposures, though it remains adequate

for *in vitro* studies. In contrast, $\Delta\psi_m$ might be an adequate biomarker of oxidant-mediated environmental stress, both *in vitro* and *in vivo*.

Materials and Methods

Reagents. We purchased paraformaldehyde and saponin from Sigma (St Louis, MO, USA), and culture medium (RPMI 1640 and Dulbecco’s modified Eagle medium), fetal calf serum (FCS), phosphate-buffered saline (PBS), L-glutamine, bovine serum albumin (BSA, fraction V), and HEPES buffer from ICN Biochemicals (Costa Mesa, CA, USA). We purchased 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyliumcarbocyanine iodide (JC-1) from Molecular Probes (Eugene, OR, USA). The monoclonal antibodies directed against the inducible form of Hsp70 [mouse immunoglobulin (Ig)G1, SPA-810] were from Stressgen (Victoria, Canada). The F(ab′)₂ fragment of rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC), used as secondary antibody, was from Dako (Glostrup, Denmark).

Cells and culture conditions. We obtained anonymous donated blood through the Assistance Publique Hôpitaux de Paris (APHP), in the form of buffy coats. We isolated monocytes by Ficoll gradient centrifugation and purified them by adherence as described previously (18). We maintained monocytes (2.5×10^5 /mL) in RPMI-1640 medium containing 10% fetal calf serum, 2 mmol/L glutamine, and 25 mmol/L HEPES.

In vitro exposure to TS. A peristaltic pump-smoke machine (Heinr. Borgwaldt RM1/G, Hamburg, Germany) generated TS-bubbled PBS from mainstream smoke of standard cigarettes (reference 2R1, University of Kentucky, Lexington, KY, USA) through a puffing mechanism mimicking the human smoking pattern (one puff = 2 sec,

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35 mL/min) (18,19). The smoke of one cigarette corresponds to 10 puffs bubbled in 5 mL of PBS, the final dilutions being expressed as puff/mL of culture medium. We incubated TS-exposed monocytes for 3 hr before analysis.

Exposure to *in vitro* γ -radiation. We performed *in vitro* γ -radiation at room temperature, in air, using a γ -ray source (^{137}Cs , irradiator IBL637) at a fixed dose rate of 2 Gy/min. We exposed cells to 5 Gy and allowed them to recover for 6, 24, 48, or 72 hr. At indicated time points, we collected irradiated and control cells and determined $\Delta\psi\text{m}$ disruption.

Detection of Hsp70 levels in human monocytes. We determined baseline levels of Hsp70 in control human monocytes by flow cytometry analysis (20). Briefly, we fixed cells for Hsp70 analysis for 10 min in paraformaldehyde 3% and then washed and incubated them with 50 μL saponin 0.6%, allowing permeabilization of the cell membrane. We detected intracellular Hsp70 with the anti-human antibody against the cytosolic inducible Hsp70 at a dilution of 1/100 in PBS with BSA at 1% (PBS/BSA) for 10 min. We removed unbound antibodies and stained cells with FITC-conjugated rabbit anti-mouse IgG at a dilution of 1/30 in PBS/BSA for 10 min before flow cytometry analysis. We performed flow cytometry on 5,000 cells/sample using an EPICS Elite flow cytometer (Coulter, Miami, FL, USA) equipped with a single 488-nm argon laser. We express baseline expression of Hsp70 as percentage of cells expressing Hsp70 and by the mean fluorescence intensity.

Determination of $\Delta\psi\text{m}$: effects of *in vitro* exposure to TS. We determined baseline $\Delta\psi\text{m}$ by staining mitochondria with JC-1

(21). The lipophilic cation JC-1 forms J-aggregates in the matrix of intact mitochondria (emitting at 590 nm) or is released in a monomeric form (527 nm) from depolarized mitochondria. Thus, mitochondrial membrane depolarization is associated with a shift in JC-1 fluorescence emission, from red to green. We suspended human monocytes in 0.5 mL of JC-1 solution (50 ng/mL in PBS) and incubated them for 10 min at 37°C in the dark before immediately analyzing them by flow cytometry. We counted 5,000 cells for each sample in acquisition and analyzed them using Elite 4.01 software; we express the results as percentage of cells with disrupted $\Delta\psi\text{m}$.

Detection of $\Delta\psi\text{m}$ disruption: relation to *in vivo* exposure to TS. Healthy, adult volunteers gave informed consent to donate 20 mL of blood. Volunteers consisted of seven nonsmokers (never smoked; three males and four females; mean age, 28 ± 6.5 years) and eight smokers (15–25 cigarettes/day; four males and four females; mean age, 28 ± 3.5 years). The study was conducted at Cochin Hospital, Paris, France, where blood samples were taken. We isolated monocytes immediately after the sampling and tested the cells to evaluate baseline levels of mitochondrial membrane depolarization.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney *U*-test.

Results

Baseline variability in Hsp70 expression and $\Delta\psi\text{m}$ in human monocytes. We performed this study in human monocytes because they are adequate for further cohort studies according to their accessibility and their high sensitivity to oxidants, especially in terms of stress protein induction (22,23). We compared interindividual variation in Hsp70 expression with that of $\Delta\psi\text{m}$ disruption in human monocytes from anonymous blood donors and performed flow cytometry, a rapid, reliable and well-adapted method for evaluating Hsp70 expression and $\Delta\psi\text{m}$ in intact cells (20,21). Although baseline levels

of any optimal biomarker to be used *in vivo* have to be stable from one individual to another, in human monocytes the percentage of cells with baseline $\Delta\psi\text{m}$ disruption ranged from 1.4% to 23.0%. The inherent variability observed for Hsp70, measured as percentage of cells expressing Hsp70, varied from 0.7% to 90%, and mean fluorescence intensity varied from 1 to 3.6 (Figure 1).

Effects of *in vitro* TS exposure on $\Delta\psi\text{m}$. We have previously shown that in human monocytes from healthy donors exposed for 3 hr to *in vitro* increasing concentrations of TS solution, $\Delta\psi\text{m}$ decreased with increasing concentrations of TS (18). Here we report on our extension of that study to a larger population. Using the same protocol, we studied over 100 donors and achieved the same results: a decrease in $\Delta\psi\text{m}$ with increasing concentrations of TS. Correlation coefficients ranging from 0.799 to 0.970 indicated a strong linear association between TS concentrations and $\Delta\psi\text{m}$. Table 1 shows 10 representative experiments.

Variability in baseline $\Delta\psi\text{m}$: relation to *in vivo* exposure to TS. Because we tested the donors anonymously, determining whether the small yet perceptible baseline variation of $\Delta\psi\text{m}$ resulted from such differences as age, sex, smoking status, or other environmental exposure was not feasible. We therefore performed another set of experiments using monocytes from volunteers with known smoking status. Baseline levels of $\Delta\psi\text{m}$ were determined in monocytes from eight young and otherwise healthy smokers and seven nonsmokers. Monocytes from smokers (representing *in vivo* TS exposure) had significantly higher $\Delta\psi\text{m}$ disruption than those from their nonsmoking counterparts. The mean percentage of cells with $\Delta\psi\text{m}$ disruption in the smoking population ($n = 8$) was $13.3\% \pm 1.3$ compared with $7.4\% \pm 0.9$ ($n = 7$) for the nonsmokers ($p < 0.05$; Figure 2).

Effects of *in vitro* γ -radiation exposure on $\Delta\psi\text{m}$ in human monocytes. $\Delta\psi\text{m}$ susceptibility to oxidants and the scarcity of reliable

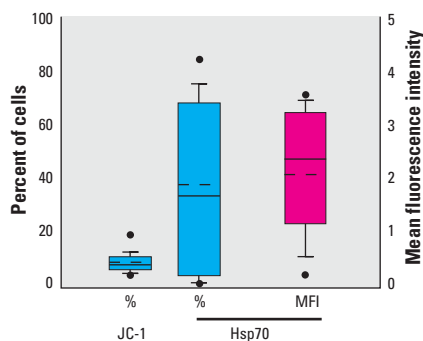


Figure 1. Variability of baseline $\Delta\psi\text{m}$ disruption compared with Hsp70 expression in human circulating monocytes: baseline $\Delta\psi\text{m}$ disruption (JC-1) and Hsp70 expression were determined by flow cytometry in human monocytes from blood of, respectively, 25 ($\Delta\psi\text{m}$) and 15 (Hsp70) anonymous healthy donors. For $\Delta\psi\text{m}$ detection, results are percentage of cells with disrupted $\Delta\psi\text{m}$; for Hsp70 expression, results are both percentage of cells expressing Hsp70 and baseline mean fluorescence intensity.

Table 1. $\Delta\psi\text{m}$ disruption as a function of *in vitro* TS exposure of human circulating monocytes.

Experiment	Control	0.03	0.06	0.12	0.18	0.24	0.3	r Value
1	11.6	18.1	13.7	55.0	20.4	97.8	98.9	0.868
2	10.5	7.5	50.7	96.5	95.7	93.1	99.4	0.856
3	5.9	6.1	7.3	21.6	48.4	66.9	73.8	0.979
4	17.7	13.4	17.1	23.4	70.7	75.8	84.0	0.943
5	8.2	23.2	17.1	20.0	27.1	31.1	75.3	0.835
6	7.6	9.3	5.9	5.6	6.6	22.0	38.0	0.799
7	10.3	12.0	10.6	22.6	21.7	22.9	37.9	0.926
8	12.2	13.5	13.9	17.3	21.9	22.0	38.1	0.914
9	23.0	11.3	25.7	35.9	58.1	87.2	80.5	0.952
10	7.3	8.3	13.3	14.5	36.5	40.8	47.4	0.970
Mean	12.5	12.3	17.3	31.2	40.7	55.9	67.3	
SEM	1.7	1.6	4.0	8.4	8.8	9.9	7.9	

Human monocytes from 10 anonymous blood donors were exposed for 3 hr to TS exposure ranging from 0.03 to 0.3 puff/mL. Monocytes were then tested for $\Delta\psi\text{m}$ disruption using flow cytometry. The equation corresponding to $\Delta\psi\text{m}$ disruption as a function of *in vitro* exposure is 19.01 with an r value of 0.68 . For the same increase in TS concentration, $\Delta\psi\text{m}$ decreased 1.9-fold compared with control.

and reproducible methods for testing radiation exposures motivated our study of the effects of another oxidant-mediated environmental exposure, ionizing radiation. We exposed human monocytes to a single dose of γ -radiation (5 Gy) and studied γ -radiation-induced modifications in $\Delta\psi_m$, 6, 24, 48, and 72 hr after radiation exposure (Figure 3, Table 2). Although $\Delta\psi_m$ did not vary significantly in unexposed cells at each recovered time, significant γ -radiation-induced $\Delta\psi_m$ disruption was detectable 24 hr after radiation ($p < 0.05$), whereas cell death evaluated as a control for radiation toxicity was detected after 48 hr ($30\% \pm 7.8$ of cell death; $n = 10$) and peaked at 72 hr (data not shown).

Discussion

In this study, we report low variability of $\Delta\psi_m$ compared with established biomarkers such as Hsp70, and a high sensitivity of $\Delta\psi_m$ disruption to oxidant exposure. Both findings favor the use of $\Delta\psi_m$ as a selective *in vitro* and *in vivo* biomarker for exposure to oxidants.

To maximize its utility, a biomarker must have the ability to respond to a large number of chemical exposures. We thus chose to study exposure to oxidants because of their significance in environmental exposure. They are indeed involved in the toxicity of many products and in the pathogenesis of many diseases (24–26). We chose TS exposure as a model for oxidative stress, first, because it has well-known effects on the health of smokers (27) and, second, because oxidants play a central role in TS-mediated toxicity and carcinogenesis (18,28–30). TS could also represent a prototype for studying the cellular response to other chemicals whose toxicity is enhanced by oxidants.

Collapse of $\Delta\psi_m$ can occur by several mechanisms independent of oxidant exposure, such as various exposure to toxins or physical damage. However, TS is a complex pollutant (it contains > 3,600 different compounds). Previous studies indicate that TS-mediated $\Delta\psi_m$ disruption relates to its oxidants content (18,31,32). Furthermore, TS induces other markers of oxidative stress

such as heme oxygenase (22,31,33), promotes lipid peroxidation, and decreases glutathione levels, thus providing direct evidence of the oxidative stress induced by TS (32,34,35). The high sensitivity of $\Delta\psi_m$ to TS exposure and the role of oxidants in TS-mediated toxicity led us to study the use of $\Delta\psi_m$ as a biomarker for oxidants.

By comparing the baseline levels of $\Delta\psi_m$ to a well-known biomarker, Hsp70, we showed that the variability of Hsp70 expression as measured by flow cytometry in human monocytes was up to 10 times higher than that of $\Delta\psi_m$ disruption. The variability in the percentage of cells expressing Hsp70 was approximately 1:130, which is the lower range of what has been described by others using the same technique or other, less sensitive methods (20,36). To be an adequately useful indicator for *in vivo* exposure, a biomarker has to demonstrate as little variability as possible from one donor to another. Thus, the inherent variability of baseline levels of Hsp70 detected in human cells renders Hsp70 expression difficult to use as a biomarker for *in vivo* exposures, though it remains a most adequate biomarker for *in vitro* and ecosystem studies. In contrast, the variability of $\Delta\psi_m$ was approximately 1:16 among 100 subjects, thus making this parameter potentially more suitable for studying the effects of *in vivo* exposures.

We have already shown that TS induces Hsp70 expression as well as mitochondrial alterations in several mammalian cells, including normal human monocytes (18,31). $\Delta\psi_m$ disruption, as an early prerequisite step toward programmed cell death (16,17), has been detected after 3 hr of exposure to TS in human monocytes, whereas cell death has not been detected before 16 hr of exposure (18,19,31), thereby meeting the criterion of sensitivity with respect to the cell death end point. Moreover, we performed detection of $\Delta\psi_m$ disruption using the lipophilic cation JC-1, chosen as a specific and sensitive probe for cytometric analysis of $\Delta\psi_m$ disruption. Indeed, it has been previously shown that JC-1 is a reliable probe for analyzing $\Delta\psi_m$ changes with flow cytometry, whereas it is

not sensitive to the depolarization of plasma membrane (37).

We tested the role of oxidant-specific mechanisms in mediating the effects of TS by preexposing cells to the antioxidant *N*-acetyl-L-cysteine (NAC) for 1 hr before TS exposure. NAC pretreatment abolished TS-mediated $\Delta\psi_m$ disruption (18). Moreover, hydrogen peroxide (H_2O_2), used at concentrations estimated similar to those found in TS, also induced $\Delta\psi_m$ disruption in a concentration-dependent manner (data not shown). In contrast, nonoxidative compounds of TS such as the carcinogen benzo[*a*]pyrene had no effect on $\Delta\psi_m$ even when used at toxic concentrations (> 50 μM) (31). Based on these data, we suggest that $\Delta\psi_m$ disruption may be an early, oxidant-specific biomarker.

Interestingly, for those donors with a high baseline percentage of cells with $\Delta\psi_m$ disruption (Figure 2; donors 2, 4, and 9), initial exposure to TS (0.03 puff/mL) had no effect on $\Delta\psi_m$, whereas those with low baseline percentage of cells with $\Delta\psi_m$ disruption (donors 1, 3, 5, 6, 7, 8) were sensitive to this low concentration of TS exposure. According to $\Delta\psi_m$ sensitivity to *in vitro* TS exposure, we anticipated that the observed differences in baseline $\Delta\psi_m$ and in the *in vitro* sensitivity to TS might be the result of voluntary *in vivo* exposure to TS: cells with relatively high levels of baseline $\Delta\psi_m$ disruption would be from smokers and cells with relatively low levels of $\Delta\psi_m$ disruption would be from nonsmokers. By testing donors with known smoking status, we corroborated the hypothesis that *in vivo* smoking status may influence $\Delta\psi_m$ because smokers had a significantly higher baseline percentage of cells with $\Delta\psi_m$ disruption than nonsmokers. The possibility that the observed differences in $\Delta\psi_m$ of smokers and

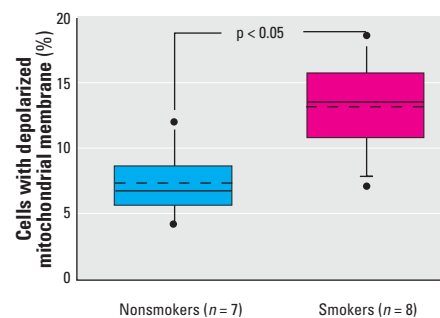


Figure 2. $\Delta\psi_m$ disruption as a function of *in vivo* TS exposure in human circulating monocytes: baseline $\Delta\psi_m$ disruption of monocytes isolated from blood of seven nonsmoker volunteer donors compared with baseline $\Delta\psi_m$ disruption of monocytes of eight smoker volunteer donors. Flow cytometer analysis revealed that nonsmokers had a mean of $7.38\% \pm 0.93$ monocytes with disrupted $\Delta\psi_m$ compared with $13.3\% \pm 1.30$ in smokers ($p < 0.05$ by Mann-Whitney *U*-test).

Table 2. Effects of γ -radiation on $\Delta\psi_m$ disruption of human circulating monocytes.

Experiment number	6 hr		24 hr		48 hr		72 hr		r-Value
	Control	20 Gy	Control	20 Gy	Control	20 Gy	Control	20 Gy	
1	5.3	4.3	4.0	27.9	5.7	33.4	61.0	50.5	0.973
2			2.0	12.3	5.4	25.9	7.3	39.9	0.997
3	15.8	15.1	16.3	33.4	17.8	44.8	17.9	45.8	0.940
4	6.8	5.8	5.4	9.3	6.9	14.5	10.3	30.2	0.958
5			8.1	14.5	8.5	36.7	10.1	57.3	0.978
6			17.3	19.1	19.8	40.1	21.9	94.2	0.895
7	6.3	7.5	7.1	26	8.1	30.8	7.4	50.3	
Mean	8.6	8.1	8.6	20.4	10.3	32.3	11.6	52.6	
SEM	2.3	2.4	2.2	3.4	2.2	3.8	2.3	7.7	

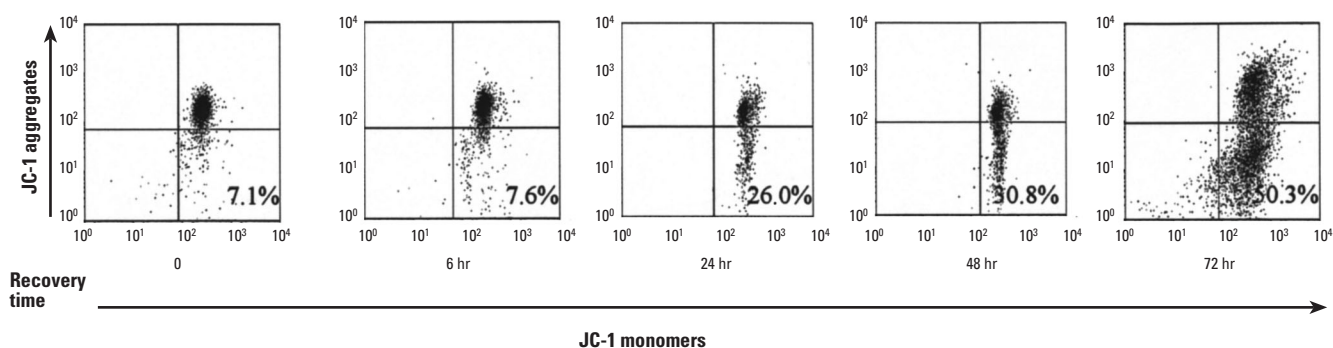


Figure 3. Effects of γ -radiation on $\Delta\psi m$ disruption of human circulating monocytes: $\Delta\psi m$ disruption of irradiated human monocytes (5 Gy) as a function of recovery time (6, 24, 48, and 72 hr). Data are from one representative experiment out of seven, which are listed in Table 2.

non smokers could be related to impaired oxidative metabolism caused by carbon monoxide should be verified. Moreover, additional experiments would test $\Delta\psi m$ in larger cohorts of subjects controlled for other environmental susceptibility factors.

Data on environmental exposures to γ -radiation are generally based on estimates of radiation-induced cancer risk derived from studies of atomic bomb survivors, irradiated victims of Chernobyl, or patients irradiated for therapeutic purposes. Data obtained with these high doses are then extrapolated for low-level exposures. The carcinogenic effect of chronic, low-level radiation exposure can be assessed from epidemiologic studies of cancer among workers in the nuclear industry. However, these studies provide only retrospective and uncertain (because of extrapolation) information rather than sensitive and rapidly detectable biomarkers of ionizing radiation. Recent research has focused on the analysis of genomic translations or other chromosome aberrations as biomarkers of radiation exposure (38–40). However, current biomarkers for radiation are still imprecise and insufficient (41). Because ionizing radiation, particularly γ , generates abundant amounts of oxidants as a result of water radiolysis (42), we tested $\Delta\psi m$ disruption as a biomarker for *in vitro* γ -radiation exposure. According to our study, $\Delta\psi m$ appears to be a sensitive and early indicator of *in vitro* radiation exposure, substantiating our other results showing $\Delta\psi m$ to be an effective early biomarker of oxidant-mediated exposures. Further studies will test the effects of *in vivo* γ -radiation exposure.

Future epidemiologic applications of $\Delta\psi m$ as a biomarker include gauging health risks associated with *in vivo* exposures to oxidant-mediated stressors such as radiation and air pollution. $\Delta\psi m$ could also be used for *in vitro* studies as a screening procedure to detect any oxidative toxicity of new industrial compounds.

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